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Relationship of vector insert size to homologous integration during transformation of *Neurospora crassa* with the cloned *am* (GDH) gene

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Summary. We used lambda and plasmid vectors containing the am^+ gene in an insert of from 2.7 to 9.1 kb, to transform *am* point mutant and deletion strains. A total of 199 transformants were examined with the potential to yield am^+ transformants by homologous recombination. When we used vectors that had 9.1 kb of homology with the chromosomal DNA, 30% of the transformants obtained were the result of homologous recombination regardless of whether the vector was a lambda molecule, a circular plasmid, or a plasmid that had been linearized prior to transformation. When vectors with up to 5.1 kb of homology were used, very few transformants (1 of 89 tested) resulted from homologous recombination. Of a sample of 29 ectopic integration events obtained by transformation with the 9.1 kb fragment cloned in a λ vector, 18 included a major part (usually almost all) of both arms of lambda with the entire *Neurospora* 9.1 kb insert between them. Four included only λ long arm sequence together with an adjacent segment of the insert containing the *am* gene. The remaining seven were the result of multiple integrations. There was no evidence of circularization of the λ vector prior to integration. All transformants that had multiple copies of the *am* gene appeared to be subject to the RIP process, which causes multiple mutations in duplicated sequences during the sexual cycle.

Key words: Homologous recombination – Transformation – *Neurospora crassa* – Ectopic integration – *R*epeat *I*nduced *P*oint mutation (RIP)

Introduction

In contrast to the situation in yeast where integrative transformation appears to occur exclusively by homologous recombination (Rine and Carlson 1985), transformation of filamentous fungi typically results in a complex variety of transformants. This includes an abundant class of transformants with the selected marker integrated at ectopic sites (frequently simultaneously at multiple sites) and those in which the selected gene has integrated, by homologous recombination, at the normal locus (for reviews see Rambosek and Leach 1987; Fincham 1989). The latter class, which is rare in most reported experiments, can take the form of a tandem duplication separated by vector sequences, or of a direct replacement of chromosomal DNA with no associated integration of vector sequences. For many kinds of studies, only events that lead to direct replacement are satisfactory.

Kinsey and Rambosek (1984) used the lambda vector λC10, (Kinnaird et al. 1982), which contains the am^+ gene as part of a 9.1 kb *Hin*dIII insert, to transform an *am* point mutant strain. They observed that about 40% of the transformants had the am^+ gene integrated near the *inl* locus on linkage group V (LGV), presumably as the result of homologous recombination at the *am* locus which is linked near *inl*. In this report we confirm the observation and show that the frequency of homologous integration at the *am* locus appears to be related to either the length of the *Neurospora* chromosomal homology provided in the insert, or a specific sequence downstream of the *am* gene. The frequency of homologous integration does not appear to be related to the form of the vector per se (i.e., linear vs circular or plasmid vs lambda). Selker and co-workers have shown that in *Neurospora* there is a process that causes multiple point mutations in duplicated DNA sequences in specialized pre-meiotic dikaryotic tissue (Selker and Garrett 1988; Selker et al. 1987). They have designated this process by the acronym, RIP (Repeat Induced Point mutation). We have confirmed their expectations that transformants with multiple copies of the am^+ gene are routinely subjected to RIP.

Materials and methods

Strains and media. The strains used in this study are shown in Table 1. *N. crassa* strains containing an *am* mutation were grown on Vogel's Minimal medium (Vogel 1964) supplemented with glutamate or alanine. Vogel's minimal medium supplemented with glycine (1.5 mg/ml) which inhibits the growth of *am* strains, was used to select for the am^+ phenotype. Crosses were made on the synthetic crossing medium of Westergaard and Mitchell (1947) using Whatman no. 1 paper as sole carbon source. The phage λC10 was propagated on *Escherichia coli* strain Q358. Plasmids were grown in *E. coli* strain JM83.

DNA mediated transformation. Conidia were made competent as described by Akins and Lambowitz (1985) and

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Table 1. *Neurospora* strains

Strain number	Known genotype	Source
74A-OR23-1VA	wild type	FGSC No. 2489[a]
J511	*am*^{6}; *al*^{s} *a*	FGSC No. 786[a,b]
J1260	*am*^{111} *cot A*	Authors stock collection[c]
J1328	*am*^{132} *inl A*	Authors stock collection[d]
J1265	*am*^{132} *a*	Authors stock collection[d]
J1664	*am*$^{23-21}$ *A*	Authors stock collection[e]
J1253	*ure-2 am*^{4}; *rec-3; cot-1 A*	Authors stock collection[f]
J1737	*am*$^{+}$	This work[g]
J1738	*am*$^{+}$	This work[g]
J1739	*am*$^{+}$	This work[g]
J1740	*am*$^{+}$	This work[g]
J1741	*am*$^{+}$	This work[g]
J1742	*am*$^{+}$	This work[h]
J1743	*am*$^{+}$	This work[i]
J1744	*am*$^{+}$	This work[j]
J1745	*am*$^{+}$	This work[g]

[a] Obtained from the Fungal Genetics Stock Center, Department of Microbiology, Immunology, and Molecular Genetics, University of Kansas Medical Center, Kansas City, KA 66103, USA

[b] The *am*^{6} allele contains a point mutation in codon 5 (Siddig et al. 1980). The *al*^{s} locus is on Linkage Group I, although the exact position has not been precisely defined

[c] The *am*^{111} allele contains a small deletion near the 3′ end of the gene

[d] The *am*^{132} strain contains a 7 kb deletion which deletes the entire coding sequence and all but approximately 1 kb from each end of the 9.1 kb *Hin*dIII fragment

[e] The *am*$^{23-21}$ allele contains a 3 kb deletion which deletes all the *am* coding sequences

[f] The *am*^{4} allele contains a point mutation near the center of the *am* coding sequence

[g] Lambda C10 transformants of *am*^{4}

[h] Plasmid pJR3 transformant of *am*^{132}

[i] *am*$^{+}$ progeny of strain J1742 and strain J1265

[j] *am*$^{+}$ progeny of strain J1743 and J1265

Vollmer and Yanofsky (1986). Transformants were picked to selective media, and allowed to grow to conidiation. The transformants were vegetatively reisolated by streaking on selective media and transferring individual microscopic conidial colonies to slants of selective media. The reisolated transformants were crossed to strains containing the *am*^{132} deletion and prototrophic progeny were isolated. Either these progeny strains or strains purified by repeated vegetative isolations were used for GDH assays and DNA isolation.

Glutamate dehydrogenase (GDH) assays. GDH assays were performed by the method of Coddington et al. (1966).

DNA isolation. Phage DNA was isolated as described by Yamamoto et al. (1970). Plasmid DNAs were isolated by the method of Clewell and Helinski (1972) and banded on CsCl gradients. *Neurospora* genomic DNA was isolated by the method of Metzenberg and co-workers (Stevens and Metzenberg 1982; Metzenberg andBaisch 1981).

DNA blots and hybridization. Two micrograms of each digested genomic DNA were fractionated by electrophoresis in 0.7% agarose gels in TRIS-acetate buffer at 15–20 volts for 16–18 h. The DNA was transferred to nylon filters (Gene Screen Plus, NEN) by capillary transfer, hybridized (1% SDS, 1M NaCl, 10% dextran sulfate, 100 mg/ml salmon sperm DNA) and rinsed (0.1 × SSC, 1% SDS, 25° C), as recommended by the manufacturer. Probes labeled with ^{32}P were produced by nick-translation or by the oligonucleotide primed labeling method of Feinberg and Vogelstein (1984). Random primers were purchased from Pharmacia.

Results

We have used vectors containing the three *am*$^{+}$ inserts shown in Fig. 1 to transform *am* mutations to prototrophy. The lambda clone, λC10, and its plasmid equivalent, pJR1, contain the *am* gene within a 9.1 kb *Hin*dIII fragment. The pJR2 and pJR3 plasmids contain the *am* gene on 2.7 kb *Bam*HI and 5.1 kb *Pst*I/*Hin*dIII fragments, respectively. As recipient strains we have used strains with *am* point mutations (*am*^{4} and *am*^{6}) as well as deletion mutations (*am*^{132}, *am*$^{23-21}$, and *am*^{111}). The locations of the mutations used and the extent of the deletions are indicated on the map of the *am* region shown in Fig. 1.

Transformations using λC10 and pJR1

Based upon the levels of GDH expression and the linkage of the incoming *am*$^{+}$ gene to the *inl* marker, Kinsey and Rambosek (1984) suggested that, when λC10 was used as a vector, some of the transformants were the result of homologous recombination events. Since this was based on indirect evidence, we wanted rigorously to test this suggestion by molecular analysis of a larger sample of transfor-

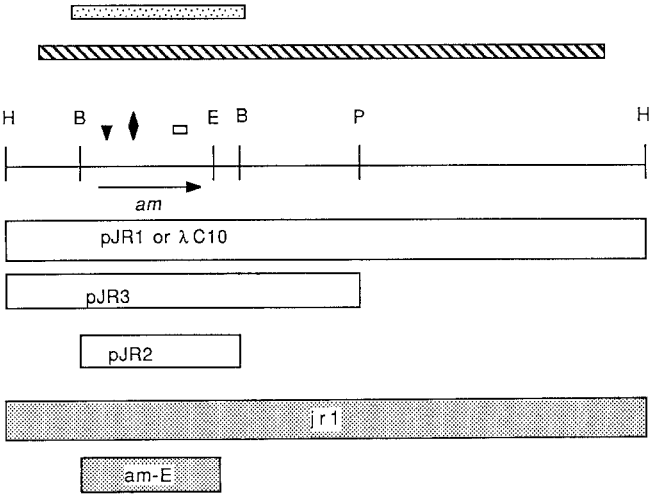

Fig. 1. Map of the 9.1 kb *Hin*dIII fragment that contains the region around the *am* gene on linkage group V (LGV) of *Neurospora*. The *thin horizontal line* represents the area pertinent to this paper. Only the restriction sites referred to in the text are indicated (H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; P, *Pst*I). The *open boxes* below the line represent the *am* region inserts present in the vectors used in this study. The *marks* and *bars* above the line indicate the extent and location of *am* mutations used (*stippled bar, am*$^{23-21}$; *hatched bar, am*^{132}; *wedge, am*^{6}; *diamond, am*^{4}; *small open box, am*^{111}). The *stippled boxes* below the bar indicate the location of the probes used in genomic Southern blots (see the Results)

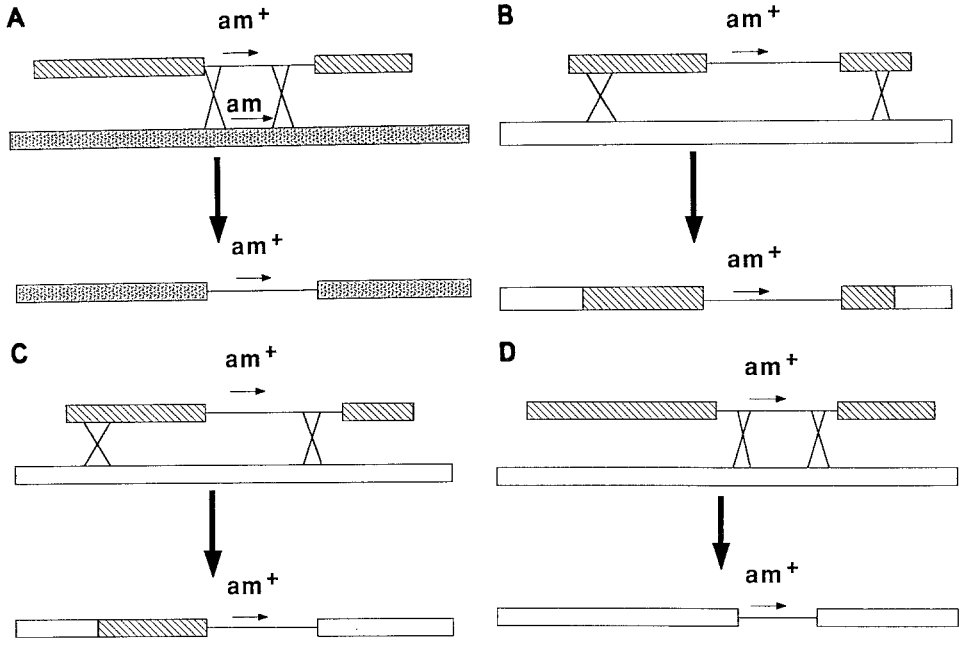

Fig. 2A–D. Possible recombination events between linear, lambda vector and *Neurospora* chromosomal DNA. **A** Homologous recombination between the incoming DNA and the *am* region of LGV. Note that no vector DNA is incorporated into the recombinant chromosome. A gene conversion event would give the same results. **B–D** Ectopic recombination of classes I–III, respectively. Note that in each ectopic integration event, a loss of some chromosomal sequences is a likely consequence

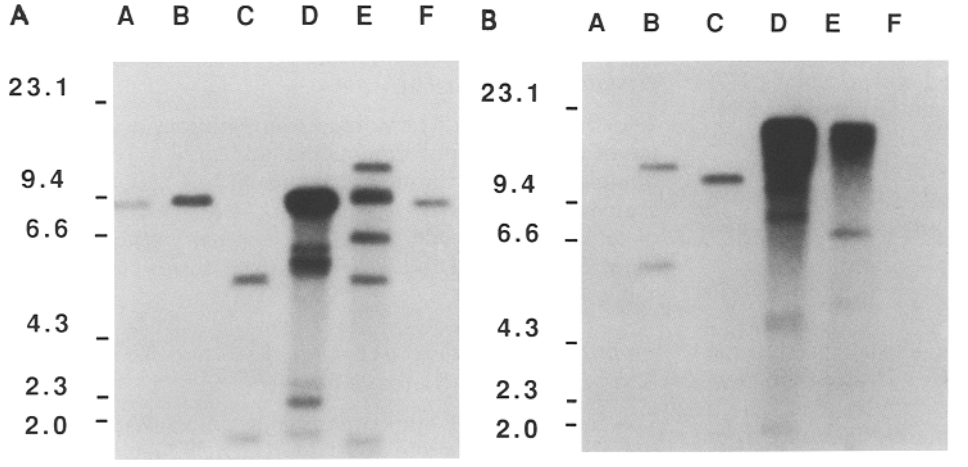

Fig. 3A and B. Genomic Southern blot of selected λC10 transformant DNAs. In both panels, genomic DNA was digested with *Hin*dIII. **A** Probed with ^{32}P labeled pJR1 (see Fig. 1); **B** probed with ^{32}P labeled lambda DNA. The strain designations for both panels read as follows: lane A, 74A-OR23-1VA; lane B, J1737; lane C J1738; lane D, J1739; lane E, J1739; lane F, J1740. Positions of size markers are given in kb

mants. We were also interested to learn whether the apparently high frequency of homologous recombination obtained with λC10 was in some way related either to the use of lambda as a vector, or to the linear nature of the transforming molecule. To test these possibilities, we used the plasmid pJR1 which contains the same am^+ insert as λC10. The plasmid was used either as a circular molecule or after linearization with restriction enzymes. We used genomic Southern blot analysis to examine a total of 39 transformants obtained using λC10 as vector and 31 transformants obtained using circular pJR1 as vector. The recipient strain in each case contained either a point mutation (am^4 or am^6) or a very small (<10 bp) deletion (am^{111}) at the *am* locus. Thus in each case there was approximately 9.1 kb of homology between the incoming DNA and the *am* region of LGV.

Possible am^+ integration events between the linear lambda vector and chromosomal DNA are shown in Fig. 2. Both homologous recombination (Fig. 2A) and ectopic integration involving recombination events in each arm of the lambda vector (Fig. 2B) would yield am^+ transformants with the am^+ gene on a *Hin*dIII fragment of 9.1 kb however, in the case of ectopic integration, Southern analysis of transformant DNA should show two bands of unknown size that hybridize to lambda probes. In contrast, if the am^+ gene has inserted by homologous recombination, there should be no bands that hybridize to lambda probes. In transformants resulting from an ectopic integration which involved one or two recombination events between the *Neurospora* sequences in the insert and non-homologous chromosomal DNA, a single new *Hin*dIII band, of unknown size, which hybridizes to the *am* probe would be expected. Those transformants in which there were two exchanges involving the *Neurospora* DNA insert (Fig. 2D) would be expected to lack lambda hybridizing bands, while those with one exchange in the insert DNA and the other in a lambda arm (Fig. 2C) would be expected to show a single band of unknown size that hybridizes to a lambda probe. A fifth pattern (not shown) would involve multiple integration of am^+ and/or lambda sequences. Southern analysis of DNA from such transformants might show any combination of the above patterns. We also considered the possibility that the *cos* ends of the lambda vector might become ligated in *Neurospora*, thus presenting the possibility that the entire

molecule might become integrated into chromosomal DNA by a single legitimate or illegitimate recombination event. Evidence, discussed below, suggest that if this occurs it is a rare event.

Figure 3 shows examples of all of the patterns of genomic Southern hybridization observed with DNA from transformants obtained with λC10 as vector. For simplicity of analysis the DNAs shown are from progeny of crosses of transformants to the deletion strain am^{132}. In each case of ectopic integration, DNA from a progeny strain with the deletion at the normal locus is shown. Use of am^+ progeny simplifies analysis and ensures homokaryosis; however, it allows the possibility that RIP has occurred. We found no evidence either at the genetic level or in genomic Southern analysis that the progeny analyzed had been effected by RIP. DNA from each transformant was digested with *Hin*dIII, subjected to electrophoresis through agarose gel, and transferred to nylon membrane as described above. The membrane was then hybridized sequentially with an *am* sequence probe (jr1 or am-E) (Fig. 1) and a probe made from total lambda L-47 DNA (L-47 is the lambda vector used to construct λC10). The membranes were stripped between probes. An example of a transformant that showed the pattern of hybridization expected for a homologous integration event is shown in lane F of each panel. A total of 11 out of 39 transformants obtained with λC10 had this pattern. When transformants obtained with circular pJR1 were analyzed, 11 of 31 showed the pattern expected for homologous integration (data not shown). Interestingly, we found no examples of homologous integration of the circular plasmid by a single exchange event. The remaining transformants appear to be the result of ectopic integration, and are discussed below.

Effect of linearization of plasmids

The effect of linearization of the plasmid sequences on the frequency of am^+ transformants was tested using the plasmid pJR1 or a derivative, pJR1-1 (which is identical to pJR1 except that a 700 bp DNA fragment originating from linkage group III has been inserted into the unique *Kpn*I site 650 bp downstream of the *am* coding sequences (Frederick et al. 1989). The plasmids were linearized with *Sma*I which cuts once in the polylinker region of pUC8, and used to transform strain J511 (am^6). A total of 27 transformants obtained with linearized pJR1 were analyzed; five gave the patterns expected for homologous recombination. Thirteen transformants obtained with linearized pJR1-1, were analyzed; four appeared to be the result of homologous recombination.

GDH expression in transformants

Kinsey and Rambosek (1984) observed that there were two classes of am^+ transformants with respect to GDH activity. The majority of transformants had 10%–20% of the normal GDH activity and were, with one exception, unlinked to the normal *am* locus. G. Fredrick and J. Kinsey (unpublished) found that the cloned am^+ gene lacks distant upstream regulatory sequences required for normal levels of GDH expression. Transformants that have resulted from homologous integration of the am^+ gene would be expected to re-establish the correct association of the gene with the upstream elements, resulting in wild-type levels of GDH expression. All of the transformants obtained with pJR1 and λC10 (a total of 97 strains) were tested for GDH expression. All of the transformants that were classified on the basis of Southern analysis as resulting from homologous recombination events showed wild-type levels of GDH expression. None of the transformants classified as resulting from ectopic integration had wild-type levels of GDH expression (Table 2).

Table 2. Relationship of integration site and glutamate dehydrogenase (GDH) activity

	Per cent of wild-type GDH activity[a]				
	0% –20%	21% –40%	41% –60%	61% –80%	81% –100%
Ectopically integrated	32	27	10	1	0
Homologously integrated	0	0	0	1	26

[a] Individual values were expressed as percentages of wild-type levels of activity. These values were assembled into the groups shown

Putative homologous transformants show linkage of the am^+ *gene to the* am *linked* his-1 *locus*

As a further test of homologous integration, a sample of ten of the λC10 transformants that appeared to result from homologous integration were tested for linkage of the integrated am^+ gene to *his-1*, which is located on LGV approximately 1 cM from the *am* locus. The am^+ gene in all of these transformants was closely linked to *his-1* (data not shown).

Transformation with vectors containing inserts with less than 9 kb of homology with the chromosome

The frequencies of homologous integration that we have observed with pJR1 and λC10 are relatively high compared to those reported in most previous studies of transformation in *Neurospora* (reviewed in Rambosek and Leach 1987; Fincham 1989). Since the *Neurospora* DNA insert in the vectors that we used was relatively large, we were interested to determine whether the frequency of homologous integration was related to the length of homology between the chromosome and the transforming plasmid. To test this we transformed *am* point mutant strains using the plasmid pJR2 which contains a 2.7 kb *Bam*H1 fragment (Fig. 1),

Table 3. Effect of vector insert size on the amount of homologous recombination

Vector	Recipient	Length of homology	Ratio of homologous insertions of total
λC10	am^{132}	2.0 kb[a]	1/19
pJR2	am^{111}	2.7 kb	0/14
pJR3	am^{23-21}	3.0 kb[a]	0/18
pJR3	am^{111}	5.1 kb	0/38
pJR1	am^4	9.1 kb	11/31
λC10	am^4, am^6, am^{111}	9.1 kb	11/39

[a] This is an interrupted stretch of homology

or the plasmid pJR3 which contains a 5.1 kb insert (Fig. 1). Two other combinations of vector and recipient strain were tried in which there was a region of interrupted homology between the vector insert and the chromosome. The phage λC10 was used to transform strain J1328 containing the am^{132} deletion. There is approximately 1 kb of homology at either end of λC10 with the chromosome in the am^{132} deletion strain. The plasmid pJR3 was used to transform strain J1664, which contains the am^{23-21} deletion. There is approximately 1.5 kb of homology at either end of the pJR3 insert and the chromosome in this strain. These transformants were analyzed by genomic Southern blots and GDH assays as described above, and the results of these experiments are summarized in Table 3. Of a total of 89 transformants examined, only one, the result of transforming strain J1328 (am^{132}) with the λC10 insert, was found to have resulted from a single homologous integration event. None of the other transformation events examined, even those where the incoming plasmid had 5 kb of homology with the chromosome, resulted in homologous integration of a single copy of am^+ sequences.

Analysis of ectopic integration events

The majority of the am^+ transformants analyzed were the result of ectopic integrations of am^+ sequences. To examine the nature of these events, DNA from 29 ectopic transformants of the phage λC10 were subjected to genomic Southern analysis using either pJR1 or the intact lambda molecule as a probe. All of these transformants (shown schematically in Fig. 2) fell into one of three classes based on the Southern patterns. Examples of genomic Southern blots for each of these three classes are shown in Fig. 3.

Class I transformants (Fig. 3, lane B) show a single 9.1 kb *Hin*dIII fragment which hybridized to the jr1 probe and two *Hin*dIII bands, of varying sizes, which hybridized to lambda sequences. Since the *am*-containing band in these transformants is the same size as the insert in λC10, this pattern suggests that the insert of λC10 was integrated still attached to the lambda arms, as shown in Fig. 2B. Interestingly, using lambda probes that came from the ends of the lambda arms, we found that in seven such strains tested, six had lambda sequences from within 2.5 kb of the end of the long arm and 5.0 kb of the end of the short arm (data not shown). This suggests that in some transformants the linear molecule was integrated, essentially intact, into the chromosome via a non-homologous recombination process. An alternate explanation for this observation might be that the *cos* ends of the lambda vector had been recircularized prior to integration. To test the latter possibility, a Southern blot of an *Acc*I/*Bcl*I digest of these DNAs was hybridized with a probe from the end of the lambda long arm. If the *cos* ends had been recircularized prior to integration this should hybridize to a diagnostic band of 2.7 kb. None of the transformants tested had such a band (data not shown). Class I transformants accounted for 62% (18/29) of the ectopic transformants examined.

DNA from class II transformants (Fig. 3, lane C), when hybridized to the jr1 probe, showed a single *Hin*dIII fragment differing in size from that of the λC10 insert. When the blots were probed with a lambda probe, a single *Hin*dIII fragment containing sequences from the long arm of lambda was seen. The observed class II transformants (which correspond to the recombination event of Fig. 2C), appear to be the result of non-homologous events between chromosomal DNA and insert sequences downstream of the incoming *am* gene on one side and lambda long arm sequences on the other side. Fourteen per cent (4/29) of the ectopic transformants analyzed were of this class.

Class III transformants (Fig. 3, lanes D and E) contained multiple bands hybridizing to both the jr1 and lambda probes, indicating the presence of multiple copies of *am* and lambda sequences. Class III transformants accounted for 24% (7/29) of the transformant strains analyzed.

Strains with multiple copies of the am *gene are subjected to RIP*

The presence of several strains with multiple integration events indicated that the presence of multiple copies of *am* sequences might, when the strains were crossed, routinely result in RIP mutations as predicted by Selker et al. (1987, 1988). Initially, it was observed that all class III transformants appeared to be very unstable through meiosis (i.e., too few am^+ spores were observed in crosses to an *am* deletion strain). This suggested that the am^+ gene(s) were being subjected to RIP. To address this question more directly, we crossed homokaryotic progeny of three class III transformants of λC10 and collected tetrads. Although it was possible to isolate rare am^+ progeny among random spores, no am^+ spores were found in the tetrads analyzed. Other markers in the crosses (*inl, cot-1*, and *mt*) showed normal segregation (data not shown). This is the result expected if the am^+ gene were being subjected to the RIP process (Selker et al. 1987, 1988). To determine if RIP was responsible for the aberrant segregation, the members of one tetrad were analyzed by Southern analysis. The progeny all showed changes in the Southern pattern with respect to the parent (data not shown).

If RIP is responsible for the poor segregation of am^+ progeny, one would expect that an am^+ gene originating in a strain that contained multiple copies of am^+ sequences would segregate normally if the copy number of the *am*

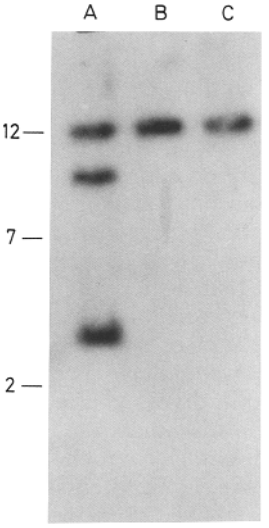

Fig. 4. Genomic Southern blot of *Hin*dIII digested DNA from transformant strain J1742 and its progeny. J1742 was obtained by transformation of J1328 with pJR3. Lane A, J1742; lane B, J1743; lane C, J1744. Positions of size markers are given in kb

gene were reduced to one. To test this hypothesis we looked at DNA from strain J1742, a pJR3 transformant of strain J1328. The original transformant made homokaryotic by streaking, contained three *am*-hybridizing *Hin*dIII bands (Fig. 4, lane A). Strain J1742 transmitted the am^+ phenotype through a cross at a low level (9%). Southern analysis of DNA from an am^+ spore showed it to contain only one of the three copies of the *am* gene that had been present in the parental strain (Fig. 4, lane B). When this strain (J1743) was crossed to the am^{132} deletion strain, J1265, the am^+ phenotype showed normal Mendelian segregation. As expected, progeny of strain J1743 also contained only one copy of the *am* gene (Fig. 4, lane C). These results were exactly as predicted by Selker et al. (1987, 1988), indicating that multiple copies of the *am* gene are subjected to RIP; however, if the gene survives the RIP process and is segregated into a nucleus devoid of other copies of the *am* gene, segregation of the single ectopic copy is normal. Fincham et al. (1989) have also made similar observations in a study of *am* transformants.

Discussion

We have used a series of am^+ vectors, containing inserts with regions of from 2.7 to 9.1 kb of homology with the *am* region of linkage group V to transform *am* mutant strains to prototrophy. As has been seen in previous reports of *Neurospora* transformation (reviewed in Rambosek and Leach 1987; Fincham 1989), regardless of the vector used, a majority of the transformants observed were the result of ectopic integration of the am^+ gene at apparently random sites. However in the present study, when vectors containing a 9.1 kb region of homology with the chromosome were used, transformants were routinely obtained that were the result of homologous recombination with a frequency of about 30%. This was independent of whether the vector was a lambda molecule, a linearized plasmid, or the supercoiled circular form of the plasmid. Obtaining transformants that result from homologous recombination at this frequency makes it feasible to target non-selectable alterations to the chromosome and locate the transformants of interest by screening by Southern analysis. We have used this approach to target a 700 bp insertion downstream of the *am* gene (Frederick et al. 1989).

When vectors containing inserts with up to 5 kb of homology with the *am* region were used, the frequency of homologous recombination was very low (1/89). There are two possible explanations for these observations. The first possibility is that there is a threshold limit of homology, below which homologous recombination is very inefficient. If this is the explanation, the threshold must fall between 5 and 9 kb. The second possibility is that there is a specific sequence that promotes homologous recombination and that this is present in the vectors with 9 kb of the *am* region and missing in the vectors with less homology. Because the *am* gene is asymmetrically placed in the 9.1 kb *Hin*dIII fragment used as the starting point in this study, the plasmids with smaller inserts have DNA only from the left half of the molecule, as shown in Fig. 1. This raises the possibility that a specific sequence contained in the region of 4 kb that is present in pJR1 but absent in the other vectors is responsible for the homologous recombination observed.

Catcheside and co-workers (Catcheside 1977) have proposed that meiotic recombination at the *am* locus is controlled by means of *cog* and *con* sites responsive to the product of the *rec-3* gene. Based on the polarity of conversion events these sites have been predicted to be upstream of the *am* gene (Smyth 1973; Fincham 1974; Rambosek and Kinsey 1983). Thus if there is a downstream sequence responsible for the homologous recombination observed in transformation, it does not correspond to any known, or predicted, element.

Kim and Marzluf (1988) reported a high frequency of homologous integration when they used a plasmid vector containing the *Neurospora trp-1* gene on a 5.1 kb insert. They observed large differences between two different recipient strains, in the frequency of homologous integration. Other than the differences due to the size of the *am* mutation (i.e., large deletion vs point mutation) we have observed only minor differences in the frequency of homologous recombination between the recipient strains that we used.

As was previously observed, the majority of transformants that we obtained were due to ectopic integration. A set of 29 transformants were analyzed, which were obtained with the lambda vector λC10 and were the result of ectopic integration. In the majority of cases (62%), the illegitimate recombination events that led to integration of the am^+ sequences were the result of recombination between chromosomal sequences and the lambda vector arms. Southern analysis with lambda probes from near the *cos* ends of the lambda arms indicated that most, or perhaps all, of the vector arms were present in the transformed strains. A search failed to find a specific 2.7 kb *Acc*I/*Bcl*I band that should be present if the *cos* ends had become ligated in the recipient strain prior to integration. This suggests that, for linear vectors, the free ends of the vector may be involved in many of the ectopic integration events in *Neurospora*. This has been proposed to be the case in the transformation of mammalian cells (Cappecchi 1989), and also indicates that *Neurospora* does not routinely catalyze the ligation of the free *cos* ends of lambda.

A minority of the transformants obtained were the result of multiple integration of *am* sequences. In each such case, very few am^+ spores were obtained when these transformants were crossed to an *am* deletion strain, which is the result predicted by Selker and colleagues (Selker et al. 1987) to result from the phenomenon of RIP. Molecular analysis supported this interpretation of the meiotic instability of these transformants.

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